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Direct Detection of Expanded Trinucleotide Repeats Using PCR and DNA Hybridization Techniques

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Recently, unstable trinucleotide repeats have been shown to be the etiologic factor in seven neuropsychiatric diseases, and they may play a similar role in other genetic disorders which exhibit genetic anticipation. We have tested one polymerase chain reaction (PCR)-based and two hybridization-based methods for direct detection of unstable DNA expansion in genomic DNA. This technique employs a single primer (asymmetric) PCR using total genomic DNA as a template to efficiently screen for the presence of large trinucleotide repeat expansions. High-stringency Southern blot hybridization with a PCR-generated trinucleotide repeat probe allowed detection of the DNA fragment containing the expansion. Analysis of myotonic dystrophy patients containing different degrees of $(CTG)_n$ expansion demonstrated the identification of the site of trinucleotide instability in some affected individuals without any prior information regarding genetic map location. The same probe was used for fluorescent in situ hybridization and several regions of $(CTG)_n/(CAG)_n$ repeats in the human genome were detected, including the myotonic dystrophy locus on chromosome 19q. Although limited at present to large trinucleotide repeat expansions, these strategies can be applied to directly clone genes involved in disorders caused by large expansions of unstable DNA.

KEY WORDS: unstable DNA, methods of direct detection

INTRODUCTION

Reports of genetic alteration in myotonic dystrophy (DM), fragile-X syndrome (Fra-X, A and E), Kennedy disease (spinal and bulbar muscular atrophy, SBMA) [Richards and Sutherland, 1992; Sutherland and Richards, 1992; Caskey et al., 1992], Huntington disease (HD) [Huntington's Disease Collaborative Research Group, 1993], spinocerebellar ataxia type 1 (SCA 1) [Orr et al., 1993], hereditary dentatorubral-pallidoluysian atrophy (DRPLA) [Koide et al., 1994; Nagafuchi et al., 1994], and Machado-Joseph disease [Kawaguchi et al., 1994; Takiyama et al., 1995] over the past 3 years have identified a new class of human mutation, referred to as trinucleotide repeat expansion. These heritable, unstable DNA sequences have emerged as an important new and unexpected concept in our perception of the human genome. This type of mutation is characterized by quantitative changes in the genotype (expansion of triplet repeats) correlating with expression of disease. The degree of DNA trinucleotide expansion may vary across different tissues of the same affected individual, and across different affected individuals of the same pedigree, especially between generations.

There is a strong body of evidence that this new mechanism of mutation may underlie several other disorders. Unstable trinucleotide repeats have been suggested as an etiological factor in spinocerebellar ataxia 2 [Pulst et al., 1993], hereditary spastic paraparesis, essential tremor, zonular cataract [Miwa, 1994], bipolar affective disorder [McInnis et al., 1993], schizophrenia [Penrose, 1984; Gindilis, 1993; Bassett and Honer, 1994; Petronis and Kennedy, 1995], and possibly autism [Ross et al., 1993]. The severity of these disorders makes the examination of an unstable DNA etiology a high research priority.

Several approaches to documenting genes containing trinucleotide repeats have been proposed. One involves selection of cloned genes containing trinucleotide repeats directly from the human genome database [Riggins et al., 1992]. A second approach screens cDNA libraries, characterizing in detail all cDNAs containing trinucleotide repeats [Li et al., 1993]. A third method of detection of trinucleotide repeats in genomic DNA (RED, repeat expansion detection) was proposed by Schalling et al. [1993].

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We have developed a set of complementary techniques which provide an opportunity for defining physical map location and can lead to the direct cloning of the unstable trinucleotide site. The three separate approaches operate independently of one another, yet can be combined for detection of unstable DNA expansion. The first approach utilizes asymmetric polymerase chain reaction (PCR) targeted towards expanded trinucleotide repeat regions. The second technique employs hybridization of a large trinucleotide repeat probe to Southern blots of digested genomic DNA from both affected individuals and controls. Lastly, fluorescent in situ hybridization (FISH) was used with the same probe to physically localize the DNA expansion.

MATERIALS AND METHODS

DNA Probe Preparation

The DNA probe for hybridization was made using the short complementary oligonucleotides $(CAG)_7$ and $(CTG)_7$. These oligonucleotides anneal to each other with a variable degree of asymmetry, and their ends remain single-stranded, thus serving as a template for Taq polymerase. PCR amplification was performed with a Perkin-Elmer Cetus 9600 thermocycler (Perkin Elmer, Norwalk, CT), using 1.0 U of Taq polymerase in the presence of 0.05 μg $(CAG)_7$ and $(CGT)_7$ oligonucleotides, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, and dTTP, 100 μM dGTP, 100 μM 7-de-aza-dGTP, and dimethyl sulphoxide (DMSO) 10%. The cycling conditions consisted of 30 cycles at 95°C for 15 sec, at 54°C for 15 sec, and at 72°C for 15 sec. Agarose gel electrophoresis showed that the size of the fragments originating from the 21-mers increased to larger than several kilobases. Radioactive labeling of 50–100 ng (1–2 μl) of the PCR product was performed using the multiprime labeling kit (Amersham Canada Ltd., Oakville, ON) and 50 μCi of ^{32}P-alpha-dCTP (5,000 Ci/mmol).

The specific probe pB750 [Surh et al., 1994] for the DM locus was labeled by random priming using the Amersham kit. Incorporation of radioactive dCTP reached 30–60%, and specific activities of the probes were 10^8–10^9 dpm/μg. Separation of unincorporated radioactive dCTP was performed on a Sephadex G-50 column, DNA grade (NICK TN Columns, Pharmacia, Uppsala, Sweden).

Asymmetric PCR

Asymmetric PCR is a substrate-dependent reaction, and so in order to reduce DNA template-related bias, concentrations of template were quantitated using spectrophotometer measurement and ethidium bromide staining on agarose gels. An asymmetric extension of a $(CAG)_7$ primer was performed using 2.0 U of Taq polymerase (Perkin Elmer Cetus) in the presence of 0.5 μg of total genomic DNA from patients affected with DM and controls, 0.5 μg $(CAG)_7$ oligonucleotides, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ 200 μM each of dATP and dCTP, 100 μM dGTP, 100 μM 7-de-aza-dGTP, and DMSO 10% in a total volume of 25 μl. The cycling conditions consisted of 150 cycles at 95°C for 30 sec, at 56°C for 60 sec, and at 72°C for 60 sec. After the first 75 cycles an additional 2.0 U of Taq polymerase were added. The PCR product was loaded on a 1.5% agarose gel and electrophoresed for 3 hr at 100 V in a standard Pharmacia LKB gel tank. The gel was blotted on a Nylon-N$^+$ membrane (Amersham) overnight.

Southern Blotting and Hybridization

Five μg of total genomic DNA from DM patients and from unaffected controls were digested with 25–30 U of restriction enzyme overnight using manufacturer's (Fermentas, Vilnius, Lithuania) recommended conditions. The digested DNA was electrophoresed on 0.8% agarose gels at 50 V for 20 hr. The gels were blotted to Hybond-N$^+$ membranes (Amersham) using 2 $\times$ SSC as transfer buffer.

Hybridization was performed at 63°C for 14–20 hr in 1.5 $\times$ SSPE, 10% SDS, 10% PEG, 100 μg/ml of herring sperm DNA, and 10–15 $\times$ 10^6 dpm of DNA probe per 20 ml of hybridization solution (one membrane). Washing of membranes containing genomic DNA was performed three times with 0.3 $\times$ SSC, 0.1% SDS at room temperature for 20 min, and then twice at 65°C for 30 min. Membranes with asymmetric PCR products were washed three times with 0.1 $\times$ SSC, 0.1% SDS at room temperature for 20 min, and then twice at 65°C for 30 min. The membranes were exposed to Kodak X-ray films for 1–3 days at -70°C with intensifying screens.

Fluorescence In Situ Hybridization

The trinucleotide repeat probe was biotinylated with dATP using the BRL BioNick labeling kit (Gibco BRL, Gaithersburg, MD). The chromosome slide was prepared by using conventional cell culture and slide-making procedures. Each patient cell line was cultured in medium at 37°C. Cells were harvested after 1 hr treatment with colcemid (0.1 μg/ml), and slides were made by using hypotonic treatment (0.4% KCl for 10 min at 37°C), fixation (methanol:acetic acid 3:1), and air-drying. The procedure for FISH was modified according to Heng et al. [1992] and Heng and Tsui [1993]. The slides were stored at 4°C for 3 days, and then baked at 55°C for 1 hr. After RNase A treatment, the slides were denatured in 70% formamide in 2 $\times$ SSC for 1 min at 70°C, followed by dehydration with ethanol. The probe was denatured at 75°C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulphate. After posthybridization washing, FISH signal detection and amplification were done according to Heng et al. [1994] and Heng and Tsui [1993].

RESULTS

Asymmetric PCR as a Provisional Screen for Large Unstable DNA Expansions

In this technique, the products of asymmetric PCR in the DNA of affected and unaffected individuals are visualized and compared. Based on the principle that a genomic DNA template that contains a relatively longer trinucleotide repeat should synthesize a longer DNA strand, we performed PCR with one complementary $(CAG)_7$ primer in DM patients and with unaffected controls.

After 150 cycles of single-primer PCR, the amplification product was run an agarose gel and blotted onto a Hybond nylon membrane (Amersham Canada Ltd.,

Oakville, ON). Subsequent hybridization with a large $(CAG)_n/(CTG)_n$ radioactive probe revealed PCR product smear pattern differences in DM patients vs. unaffected controls (Fig. 1). There are two critical innovations in this approach. First, in the asymmetric PCR, the goal is to have an elongation of the primer specifically on the expanded trinucleotide repeat template and not into the flanking region. A simple way to achieve an isolated synthesis in the repeat region is to use only the three dNTPs which correspond to the constituents of the primer (e.g., dATP, dCTP, and dGTP, if the primer used is $(CAG)_7$). Absence of one dNTP inhibits DNA synthesis as soon as the complementary nucleotide to the one missing is encountered in the template sequence.

The second innovation in our technique is related to potential interference by the template DNA on the hybridization signal. Since 0.5–1.0 μg of DNA template is required for asymmetric PCR, even nonexpanded trinucleotide loci produce a strong signal of hybridization which interferes with the newly synthesized single-stranded DNA. In our experience, the simplest way to circumvent this problem is to directly transfer the gel to the nylon membrane without prior denaturation, preventing hybridization to the predominantly double-stranded DNA template.

In the asymmetric PCR, the clearest differences could be seen for individuals with large expansions, as long as 0.5 kb or more. For this degree of expansion, we were able to reliably discriminate among approximately 70% of tested individuals severely affected with DM (N = 20). DNA samples with < 0.5 kb expansion failed to show differences in amplification products from the control samples and/or were inconsistent from sample to sample, and from experiment to experiment. An explanation for the overlap in signal between DM samples and unaffected controls is that controls have many relatively large trinucleotide repeat sites in their genomes, which might act as a template region for PCR primer annealing.

In contrast to $(CAG)_n$ expansions, asymmetric PCR does not appear usable for detection of the $(CCG)_n/(CGG)_n$ repeats. The presence of both dCTP and dGTP allows the extension of two imperfectly annealed $(CCG)_7$ oligonucleotides, and therefore the synthesis of a complementary $(CGG)_n$ strand. For this reason, oligonucleotide dimerization and extension dominate over the asymmetric synthesis from the expanded trinucleotide region. In this case, the RED technique [Schalling et al., 1993] or the Southern blot-based approach is indicated.

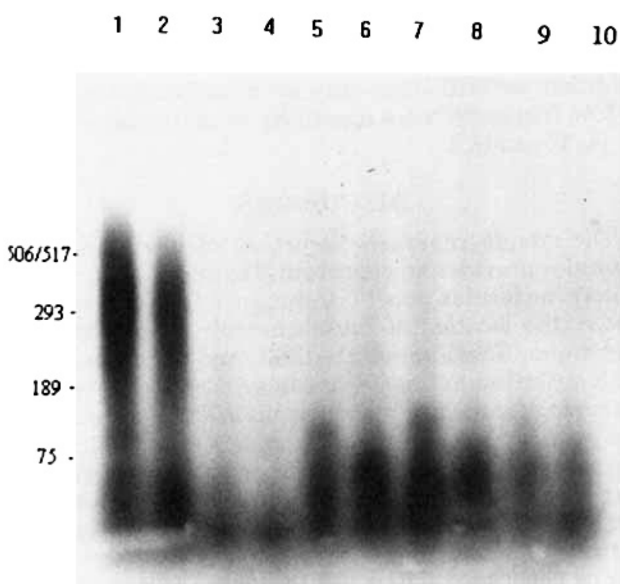


Fig. 1. Southern blot analysis of asymmetric PCR-amplified genomic DNA from DM-affected individuals. Genomic DNA was amplified with one complementary $(CAG)_7$ primer, and the PCR products were separated by electrophoresis on a 1.5% agarose gel, transferred onto a nylon membrane, and hybridized with a radiolabelled $(CAG)_n/(CTG)_n$ probe. In DM-affected individuals (**lanes 1 and 2**), the degree of expansion is 2.0 and 1.4 kb, respectively. The PCR product from these individuals shows a distinct large smear of the hybridization signal. In contrast, in control individuals (**lanes 3–10**), the hybridization signal is smaller in area, representing nonexpanded $(CAG)_n/(CTG)_n$ loci. At left, a double-stranded DNA size marker is provided (bp).

Southern Blotting for Identification of DNA Restriction Fragments Containing an Expanded Trinucleotide Repeat

The use of Southern blotting for direct detection of unstable DNA expansion is based on three premises. First, the degree of expansion in unstable DNA disorders varies between several hundred and several thousand nucleotides in length, and thus is in the range of efficient separation on agarose gels. Second, given the proper conditions, a simple trinucleotide repeat probe per se should be able to detect the genomic DNA fragment containing the expansion. Third, the number of trinucleotide loci of significant length in the genome is limited. For $(CAG)_n/(CTG)_n$ repeat-containing genes, the number was estimated to be in the range of 40–100 [Caskey et al., 1992; Li et al., 1993].

We applied a Southern blotting technique for the detection of $(CTG)_n/(CAG)_n$ amplification and evaluated its ability to identify DM-affected individuals. In the first series of experiments, we used a nonspecific $(CAG)_n/(CTG)_n$ probe for hybridization with DM DNA blots prepared under standard conditions, for characterization of large non-PCR-able DM expansions. Even when a DNA probe with a sequence specific for the DM locus is applied, the detection of large trinucleotide expansion by Southern blot hybridization is rather complicated due to somatic mosaicism, i.e., a variable degree of expansion in the cells of the same tissue of the same individual. The efficiency of the detection of these somatically variable DNA expansions can be increased by the application of relatively rare cutting enzymes. *Eco*RI cuts a large 9–10 kb fragment containing the unstable DNA region in the DM gene, and the fragment length reduces the smearing effect of the somatic variability in the length of the unstable DNA. To detect allele size differences between affected and unaffected individuals in our study, more than 20 hr of gel electrophoresis on a low-concentration (0.6–0.7%) agarose gel was required. In this series of experiments, the majority of the 20 DNAs tested from individuals affected

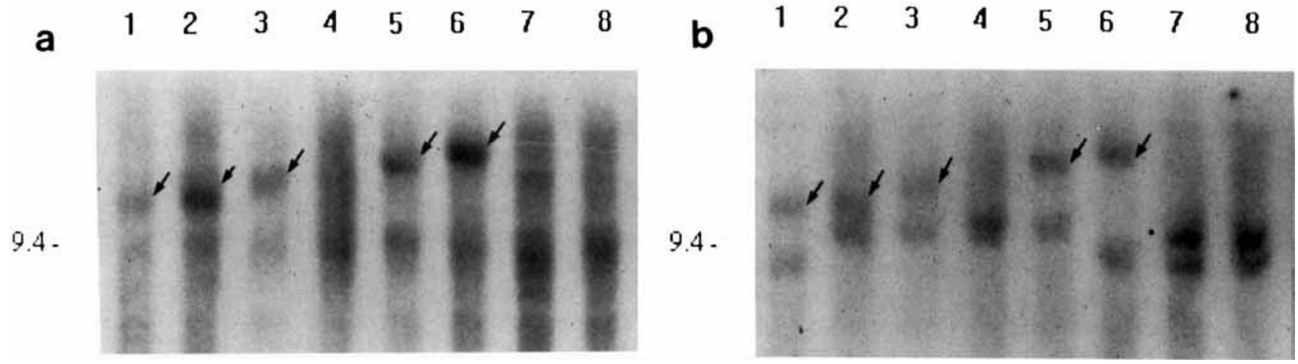


Fig. 2. Southern blot analysis of DM-affected and unaffected individuals using a trinucleotide repeat probe and a specific DM probe. **a:** DNA samples from DM patients and unaffected controls were digested with *Eco*RI. Hybridization with the PCR-generated trinucleotide repeat probe revealed the presence of $(CTG)_n/(CAG)_n$-containing bands. Hybridization bands of increasing size from 10–14 kb were observed in affected individuals (**lanes 1–3** plus **5 and 6;** indicated by arrows). In the unaffected individuals (**lanes 4, 7, and 8**), no bands similar in size to the expanded alleles were detected. **b:** The specific DM probe pB750 detects the same expanded DNA fragments plus the normal alleles.

with DM with large DNA expansions were identified with a $(CTG)_n/(CAG)_n$ repeat probe. The sensitivity threshold of the nonspecific DNA probe was about 0.6–0.8 kb; DNA templates with a lower degree of expansion were difficult to differentiate from controls. Figure 2a shows several DNAs from unrelated DM patients digested with *Eco*RI and hybridized with the $(CTG)_n/(CAG)_n$ repeat probe. The expanded DM alleles were verified in a second hybridization with the specific DM probe pB750 (Fig. 2b).

In the second set of experiments, we simulated conditions that would exist when optimal restriction enzyme and gel electrophoresis conditions were unknown in the screening for new unstable DNA sites. Two DM samples, one with a 2.2-kb somatically-stable expansion, and one with a 1.6-kb somatically-mosaic $(CTG)_n$ expansion, were selected. The potential for any of five restriction enzymes (*Eco*RI, *Pst*I, *Hind*III, *Bst*NI, and *Taq*I) to identify different degrees of DNA expansion was explored. A radioactive $(CTG)_n/(CAG)_n$ repeat probe was used for hybridization. A DM allele with a 2.2-kb length expansion was clearly revealed in three out of five digests (using the enzymes *Pst*I, *Bst*NI, and *Taq*I) (Fig. 3a,b). For the other two enzymes (*Eco*RI and *Hind*III), several hybridization bands of greater intensity were identified as well, although this finding was not as clear as for the previous three enzymes (Fig. 3a).

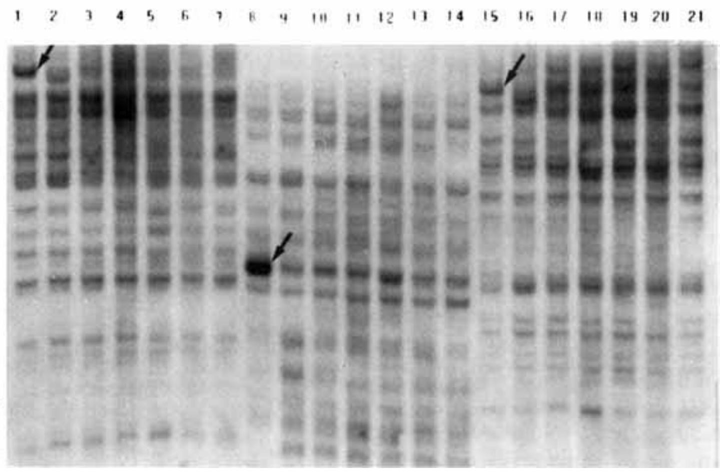
The analysis of the somatically-mosaic DNA expansion was more complicated, although some of these expansions can also be detected under regular gel electrophoresis conditions. The presence of somatically-variable DNA expansions (average length of 1.6 kb) was suspected in the *Bst*NI digestion (Fig. 3b). Two relatively intense bands (2.0 kb and 1.7 kb) were observed in the affected individual, while the unaffected controls showed fewer and less intense hybridization bands in that size range of the autoradiogram. Verification of the DM locus for all enzymes was performed by direct hybridization with a DM-specific probe, pB750, which covers the trinucleotide repeat region (data not shown).

Fluorescence In Situ Hybridization for Physical Mapping of Large Trinucleotide Repeats

We have tested the ability of FISH technology to localize $(CAG)_n/(CTG)_n$ expansion on the chromosomes of the DM patient with an expansion of 2.2 kb. Using the PCR-produced $(CAG)_n/(CTG)_n$ probe, a clear hybridization signal on the long arm of chromosome 19, consistent with the location of the DM locus, was identified (Fig. 4). A total of 35 mitotic figures were photographed, and the frequency of the positive region for the FISH signal was recorded. Approximately 10% of mitoses showed a positive signal at the DM locus on 19q13.2. In addition, several other sites of hybridization signal at <10% frequency were identified on chromosomes 1, 3, 8, 11, 15, and 16.

DISCUSSION

The simple repetitive structure of unstable trinucleotides provides an opportunity to apply a set of well-known molecular genetic techniques in an attempt to detect the location of these unstable sites. The RED technique [Schalling et al., 1993] and the asymmetric PCR described in this paper can be used for provisional screening for the presence of expanded trinucleotides in the genomic DNA of a large number of individuals. If the results of this screening are positive, Southern blotting would be the next step in the characterization of the locus of expansion. To amplify and clone a region of trinucleotide repeats, PCR with degenerate primers can be performed. There are three sets (nine permutations) for a "trinucleotide-end" PCR primer: 1) $(XYZ)_nN$ (where XYZ is a given trinucleotide, and N is not X); 2) $(XYZ)_nXN$ (where N is not Y); and 3) $(XYZ)_nXYN$ (where N is not Z). There are at least three options for selection of the second primer: 1) an oligonucleotide complementary to the interspersed repetitive sequence (IRS-PCR) [Nelson et al., 1989; Cotter et al., 1990, 1991; Ledbetter et al., 1990]; 2) a mix of degenerate oligonucleotide primers (DOP-PCR) [Telenius et al., 1992; Zhang et al., 1992; Ruano et al., 1993]; and 3) ligate DNA fragments



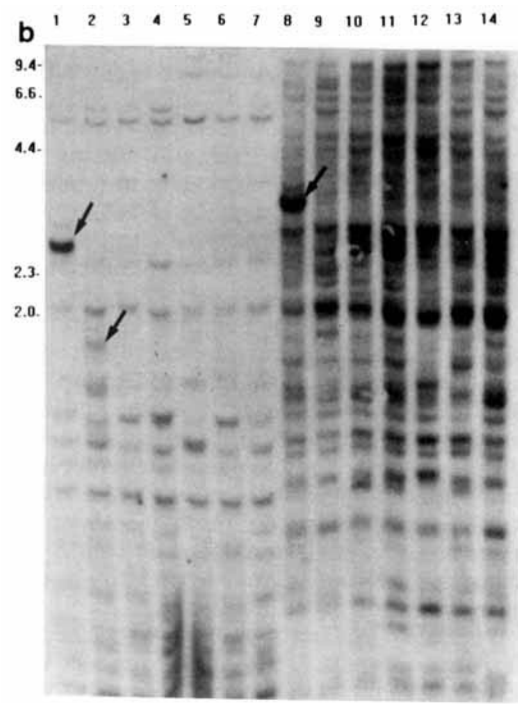


Fig. 3. **a:** Southern blot analysis of DM-affected and -unaffected controls using a trinucleotide repeat probe. DNA was analyzed from 7 individuals, 1 with a somatically-stable 2.2-kb expansion (**lanes 1, 8, and 15**), 1 with a somatically-mosaic expansion with an average length of 1.6 kb (**lanes 2, 9, and 16**), plus 5 unaffected controls (**lanes 3–7, 10–14, and 17–21**). DNA was digested with restriction enzymes *Eco*RI (**lanes 1–7**), *Pst*I (**lanes 8–14**), and HindIII (**lanes 15–21**). The DM allele with the somatically-stable expansion was clearly revealed in the *Pst*I digest (**lane 8**, indicated by arrow). **b:** In Southern blot hybridization analysis, DNA was digested with restriction enzymes *Bst*NI (**lanes 1–7**) and *Taq*I (**lanes 8–14**) in DM-affected individuals (**lane 1:** stable 2.2 kb expansion; **lane 2:** somatically-mosaic expansion, 1.6 kb average size) and unaffected controls (**lanes 3–7 and 10–14**). The DM allele with the somatically-stable expansion was evident for both restriction digests (**lanes 1 and 8**, indicated by arrows). In the case of the *Bst*NI digestion, somatically-mosaic DNA expansion was presumed to correspond to the bands of approximately 1.7–2.0 kb, and this was supported by hybridization with the specific DM probe pB750 (data not shown).

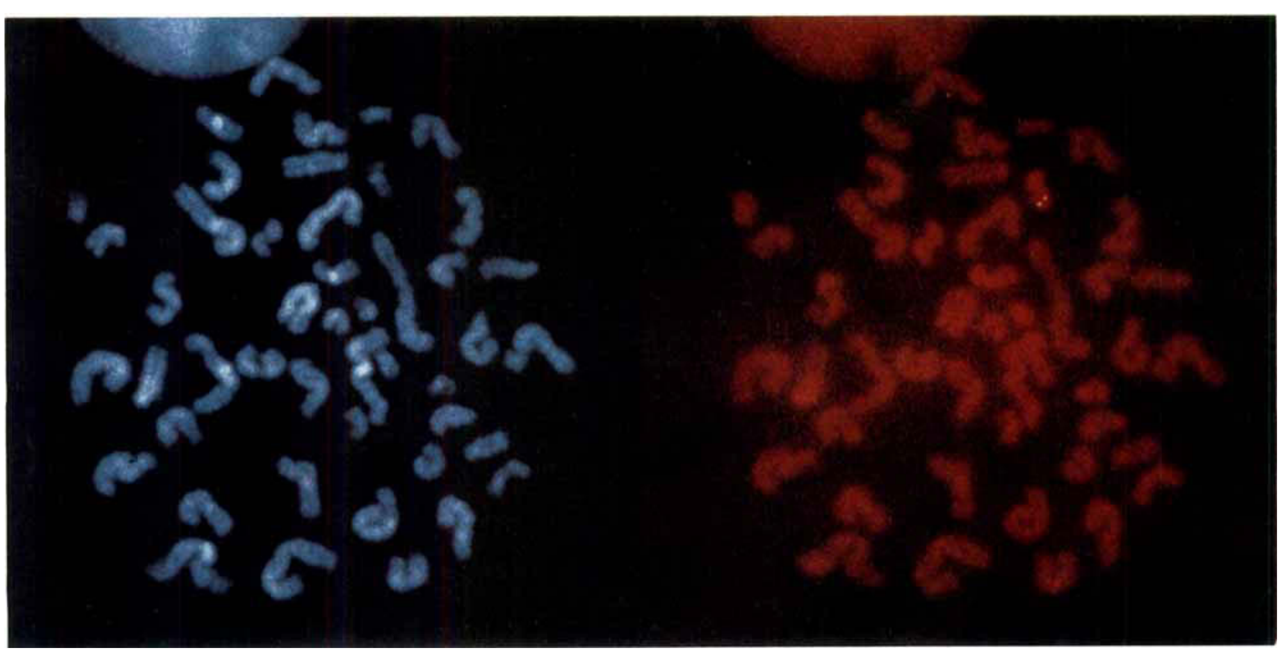


Fig. 4. Fluorescent in situ hybridization (FISH) for direct detection of $(CTG)_n/(CAG)_n$ expansion in a DM-affected individual. **Right:** FISH signals (paired yellow spots) are visible on one of the two chromosome 19 long arms in a location consistent with the DM locus. The chromosomes are stained with propidium iodide. **Left:** Same mitotic figure, stained with DAPI for individual chromosome identification.

potentially containing unstable repeats and short oligonucleotides which serve as the annealing site for a second primer in PCR, or linker adaptor-PCR [Ludecke et al., 1989; Saunders et al., 1989].

In the selection of restriction enzymes used for digestion of genomic DNA, frequent cutting enzymes are likely to be the most informative and useful. However, somatic mosaicism of unstable DNA may increase the signal variation in conjunction with these frequent cutting enzymes. Therefore, the most efficient approach to demonstrating expansion may be a combination of different restriction enzymes plus variation in gel electrophoresis (gel concentration and electrophoresis duration). It is important to analyze a group of individuals with different degrees of severity, and thus likely to have different degrees of somatic variability, in view of the fact that it is much easier to detect nonmosaic expansions compared with mosaic ones. A simultaneous analysis of unaffected family members (relatives) is also recommended, because they would provide additional information about identical-by-descent, but disease-unrelated, hybridization signals.

For exact localization of the region of expansion on the physical map, FISH can be applied. When there is strong asymmetric PCR or RED evidence for the presence of expansion, but, due to somatic mosaicism, it is difficult to localize the DNA band with Southern blotting, FISH detection may be the only option to physically map the expansion.

The methods presented here do not have sufficient resolving power to detect small expansions such as those found in HD or SCA1. They are, however, fully applicable for the detection of large expansions as seen in DM or Fra-X. Successful detection of currently uncharacterized trinucleotide repeat expansions is likely to come from the use of a variety of strategies, including the PCR- and Southern blot hybridization-based methods.

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